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(71) Applicants: NOVOZYMES A/S [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd (DK). CHR. HANSEN A/S [DK/DK]; Boege Alle 10-12, DK-2970 Hoersholm (DK).

(72) Inventors: STRINGER, Mary, Ann,; Soeborg Hovedgade 39 C, 3.tv., DK-2860 Soeborg (DK). FATUM, Tine, Muxoll,; Soevangen 50, DK-3450 Alleroed (DK). PATKAR, Shamkant, Anant,; Christoffers Allé 91, DK-2800 Lyngby (DK).

(74) Common Representative: NOVOZYMES A/S; Patents, Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

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(54) Title: PHOSPHOLIPASE AND METHOD OF PRODUCING IT

(57) Abstract: The present invention is related to a method for producing a phospholipase by processing an expressed fungal peptide and to certain specified phospholipases. Furthermore the invention provides a method for producing cheese with a phospholipase.

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PHOSPHOLIPASE AND METHOD OF PRODUCING IT

FIELD OF THE INVENTION

The present invention relates to a method of hydrolyzing a phospholipid, a method of producing a phospholipase, a method of making cheese, and to a phospholipase.

BACKGROUND OF THE INVENTION

Soragni, E., et al. (2001) EMBO J. 20: 5079-5090 discloses a phospholipase (TbSP1) from *Tuber borchii* and the nucleotide sequence of a cDNA of a gene encoding it. The following peptide sequence are published in the indicated sources, derived from the indicated source organism:

- COGEME Phytopathogenic Fungi and Oomycete EST Database, Unisequence ID: VD0100C34, *Verticillium dahliae.*
- NCBI Protein database, gi:18307435, *Neurospora* crassa
- NCBI Protein database, gi:16519372, *Helicosporum sp.* HN1
- WO 0056762, SEQ ID NO: 5954, *Aspergillus oryzae*
- TREMBL Protein database, EAA28927, *Neurospora crassa*

US 6399121 discloses the use of phospholipase in cheese making.

SUMMARY OF THE INVENTION

The inventors have analyzed known sequence data for fungal Group XIII phospholipases A2, and they have identified additional sequences, either from published sequence data or by screening for relevant sequences from natural sources. By expressing genes encoding fungal Group XIII phospholipases A2 in a suitable host organism they found that the expressed sequences consist of a core peptide coupled to a peptide sequence at the N- or C-terminal side, or both, and that expression of the gene in a suitable host organism can lead to cleavage of the expressed peptide to obtain the core peptide without any peptide extension at the N- or C-terminal. They further found that the core peptide without any peptide extension(s) has a significantly higher phospholipase activity than the core peptide linked to the peptide extension(s). Finally, they found that the core peptide discovered by this method is similar in length and sequence to a known mature peptide from *Helicosporium sp.* (Wakatsuki, S. et al. (2001) Biochim. Biophys. Acta 1522: 74-81) of unknown function, and to bacterial Group XIII phospholipases A2, which lack peptide extensions other than secretion signals (Sugiyama, M. et. al. (2002) J. Biol. Chem. 277:20051-20058).

The inventors additionally found that phospholipase sharing the active site sequence similarity and cysteine residue conservation of fungal Group XIII phospholipase A2 is useful in

cheese making.

Additionally, the inventors discovered and isolated a gene encoding a novel phospholipase from *Fusarium venenatum* A3/5, which was originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, Fungal Genetics and Biology 23: 62-80; and O'Donnell et al., 1998, Fungal Genetics and Biology 23: 57-67. The phospholipase belongs to the fungal/bacterial group XIII PLA2 as defined by Soragni et al., The EMBO Journal, 20 (2001), 5079-5090. The inventors also cloned the novel phospholipase encoding gene into an *E. coli* strain, and used the cloned gene to make a construct for expressing the *Fusarium* phospholipase gene in *Aspergillus oryzae*. The inventors transformed *Aspergillus oryzae* with this construct, and isolated the phospholipase from transformed *Aspergillus* cells.

Accordingly, the invention provides a method of producing a phospholipase which comprises processing an expressed fungal peptide so as to cleave off a peptide from the C-terminal end and/or a peptide from the N-terminal end to obtain a core peptide, wherein the core peptide comprises:

a) the amino acid sequence given by amino acids 146-153 of SEQ ID NO: 1, amino acids 87-94 of SEQ ID NO: 3, or amino acids 79-86 of SEQ ID NO: 12; or a sequence identical to any of these amino acid sequences except for the substitution of a single amino acid with another amino acid; and

b) at least two cysteine residues located on the N-terminal side of the sequence given in a); and

c) at least two cysteine residues located on the C-terminal side of the sequence given in a).

The invention also provides a method for hydrolyzing a phospholipid with a phospholipase of the invention. Furthermore the invention provides a method for producing cheese by contacting cheese milk or a fraction of cheese milk with a phospholipase and producing cheese from the cheese milk.

Finally, the invention provides phospholipase which is a polypeptide having an amino acid sequence which is at least 80 % identical with certain specified sequences.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an alignment of amino acid sequences of fungal group XIII phospholipases A2, showing processing sites (|) where known. The active site consensus is underlined. Conserved cysteine residues are indicated with | under the consensus. Alignment was made with the AlignX program of the Vector NTI program suite v8. The algorithm used is ClustalW with the blosum62mt2 matrix and AlignX default settings.

DETAILED DESCRIPTION OF THE INVENTION

Expressed peptide

The invention uses an expressed fungal peptide belonging to a group defined by the active site sequence similarity and cysteine residue conservation used in the definition of the group "fungal/bacterial group XIII phospholipase A2" given by Soragni, E., et al. (2001) EMBO J. 20: 5079-5090. The peptide is fungal, e.g. derived from *Tuber*, *Verticillium*, *Neurospora*, *Helicosporum*, or *Aspergillus*, particularly *T. borchii*, *T. albidum*, *V. dahliae*, *V. tenerum*, *N. crassa*, *Helicosporium sp.*HN1 or *A. oryzae*.

The peptide may have phospholipase activity, e.g. phospholipase A activity, such as phospholipase A1 and/or phospholipase A2 activity.

Some particular examples are known peptides having amino acid sequences listed in the sequence listing as follows. The source organisms and literature references are also indicated:

- SEQ ID NO:1. *Tuber borchii.* Soragni, E., et al. (2001) EMBO J. 20: 5079-5090
- SEQ ID NO: 3. *Verticillium dahliae.* COGEME Phytopathogenic Fungi and Oomycete EST Database, Unisequence ID: VD0100C34.
- SEQ ID NO: 4. *Neurospora* crassa. NCBI Protein database, gi:18307435.
- SEQ ID NO: 5. *Helicosporum sp.* HN1. NCBI Protein database, gi:16519372.
- SEQ ID NO: 7. *Aspergillus oryzae.* WO 0056762, SEQ ID NO: 5954.
- SEQ ID NO 8. *Neurospora crassa.* TREMBL Protein database, EAA28927

Further, the following fungal phospholipases having the indicated sequences were isolated by the inventors from natural sources purchased from public collections or collected in the indicated country and year:

- SEQ ID NO: 10. *Tuber albidum.* Purchased from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, isolate CBS272.72
- SEQ ID NO: 12. *Verticillium tenerum.* Ireland, 1996

The inventors inserted the gene from *T. albidum* (SEQ ID NO: 9) into *E. coli* and deposited the clone under the terms of the Budapest Treaty on the 12 February 2003. The deposit was made at the Deutsce Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany, and was accorded deposit number DSM 15441.

In one embodiment the invention provides a phospholipase which is a polypeptide having an amino acid sequence which is at least 80 %, such as at least 85%, preferably 90%, more preferably at least 95%, identical with amino acids 91-210 in SEQ ID NO: 10 (*T. albidum*), amino acids 92-211 in SEQ ID NO: 1 (*T. borchii*), amino acids 30-137 in SEQ ID NO: 12 (*V. tenerum*), amino acids 38-145 in SEQ ID NO: 3 (*V. dahliae*), amino acids 44-151 in

SEQ ID NO: 4 (*N. crassa*), amino acids 37-157 in SEQ ID NO: 7 (*A. oryzae*), or amino acids 58-168 in SEQ ID NO: 8 (*N. crassa*).

Peptide processing

By analyzing the phospholipase sequences in the sequence listing, the inventors found that each expressed amino acid sequence consists of a signal peptide, a core peptide, and additionally a peptide sequence with unknown function attached to the C- or N-terminal, or both, of the core peptide.

Core peptide

The core peptides are characterized by the same active site sequence similarity and cysteine residue conservation observed by Soragni, E., et al. (2001) EMBO J. 20: 5079-5090 for the fungal/bacterial group XIII phospholipase A2.

In a preferred embodiment of the invention the core peptides comprises: a) the sequence given by amino acids 146-153 of SEQ ID NO: 1, amino acids 87-94 of SEQ ID NO: 3, or amino acids 79-86 of SEQ ID NO: 12; or a sequence identical to any of these amino acid sequences except for the substitution of a single amino acid with another amino acid; and b) two cysteine residues located on the N-terminal side of the sequence given in a); and c) two cysteine residues located on the C-terminal side of the sequence given in a).

One of the cysteine residues located on the N-terminal side of the sequence given in a), may e.g. be separated from the sequence given in a) by 0-5 amino acids, such as 0-3 amino acids, preferably 0-2 amino acids, and even more preferably 1 amino acid. Another of the cysteine residues located on the N-terminal side of the sequence given in a) may e.g. be separated from the sequence given in a) by 14-20 amino acids, such as 15-19 amino acids, preferably 16-18 amino acids, and even more preferably 17 amino acids.

One of the cysteine residues located on the C-terminal side of the sequence given in a), may e.g. be separated from the sequence given in a) by 22-29 amino acids, such as 23-28 amino acids, preferably 24-27 amino acids, and even more preferably 25-26 amino acids. Another of the cysteine residues located on the C-terminal side of the sequence given in a) may e.g. be separated from the sequence given in a) by 27-49 amino acids, such as 29-46 amino acids, preferably 30-43 amino acids, even more preferably 32-42 amino acids, and most preferably 35-40 amino acids.

In a preferred embodiment the core peptide comprises four cysteine residues aligning with the cysteine residues of SEQ ID NO:1 with amino acid numbers 128, 144, 180, and 194, respectively, when the complete expressed phospholipase sequence is aligned simultaneously with the sequences given in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

According to the invention, the expressed polypeptide is cleaved so as to separate the core peptide from the attached peptide(s). The cleavage may be done *in vivo* by expressing it in a suitable filamentous fungal host or *in vitro,* e.g. by a treatment with a suitable protease such as e.g. Kex2.

The cleavage points may be found within 11 amino acids of a sequence which is FG or within 10 amino acids of a sequence which is a Kex2 site. Kex2 sites are e.g. RR, KR, KK or RK. In one embodiment the core peptide has a length of 100-150 amino acids, such as 110-140 amino acids, 115-133 amino acids, 118-129 amino acids, or 118-126 amino acids.

In one embodiment of the invention the expressed phospholipase is cleaved within 0-18 amino acids, such as 3-16 amino acids, preferably 5-14 amino acids on the N-terminal side of the sequence aligning with amino acids 97-101 of SEQ ID NO: 1, when the complete expressed phospholipase sequence is aligned simultaneously with the sequences given in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

In a preferred embodiment the expressed phospholipase is cleaved within 0-11 amino acids, such as 0-9 amino acids, preferably 0-7 amino acids, on the C-terminal side of the sequence aligning with amino acids 204-209 of SEQ ID NO: 1, when the complete expressed phospholipase sequence is aligned simultaneously with the sequences given in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

In a preferred embodiment the processed phospholipase has a specific phospholipase activity, which is higher than the activity of the expressed peptide before processing, e.g. in one embodiment the specific phospholipase activity is at least 2 times, more preferably at least 5 times, most preferably at least 10 times the specific phospholipase activity of the expressed peptide before processing. In one embodiment of the invention the expressed peptide does not have measurable phospholipase activity before processing.

Phospholipase activity may e.g. be measured in the LEU assay by hydrolyzing soy lecithin (L-alfa-phosphotidyl-choline) at pH 8.0 and 40 °C for 2 minutes. Phospholipase activity is expressed as the rate of titrant consumption (0.1 M NaOH) necessary for keeping constant pH, relative to a standard.

Expression in filamentous fungal host cell

The filamentous fungal host cell may e.g. be a cell of *Acremonium, Aspergillus, Fusarium, Humicola, Myceliophthora, Neurospora, Penicillium, Rhizomucor, Thermomyces, Thielavia, Tolypocladium,* or *Trichoderma, particularly A. awamori, A. foetidus, A. japonicus, A. nidulans, A. niger, A. oryzae. F. bactridioides, F. cerealis, F. crookwellense, F. culmorum, F. graminearum, F. graminum, F. heterosporum, F. negundi, F. oxysporum, F. reticulatum, F.*

roseum, F. sambucinum, F. sarcochroum, F. sporotrichioides, F. sulphureum, F. torulosum, F. trichothecioides, F. venenatum, H. insolens, M. thermophila, N. crassa, P. purpurogenum, R. miehei, Thermomyces lanuginosus, Thielavia terrestris, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or *Trichoderma viride.*

In a preferred embodiment the host organism is a strain of *Aspergillus, Fusarium,* or *Trichoderma,* particularly *A. niger, A. oryzae, F. venenatum, F. sambucinum* or *F. cerealis*

The transformation, cultivation, expression, recovery may be performed by conventional methods, e.g. by the general methods described in EP 238023, EP 305216, WO 9600787, EP 244234 or T. Christensen et al., BioTechnology, vol. 6, Dec. 1988, 1419-22.

Phospholipase polypeptide and DNA

In one embodiment, the present invention relates to polypeptides having phospholipase activity and where the polypeptides comprises, preferably consists of, an amino acid sequence which has a degree of identity to amino acids 29 to 149 of SEQ ID NO: 16 (i.e., the mature polypeptide) of at least 80%, such as at least 85%, even more preferably at least 90%, most preferably at least 95%, e.g. at least 96%, such as at least 97%, and even most preferably at least 98%, such as at least 99%.

Preferably, the polypeptides comprise the amino acid sequence of SEQ ID NO: 16; an allelic variant thereof; or a fragment thereof that has phospholipase activity. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 29 to 149 of SEQ ID NO: 16. In a further preferred embodiment, the polypeptide consists of amino acids 29 to 149 of SEQ ID NO: 16.

The present invention also relates to a polynucleotide comprising, preferably consisting of, a nucleotide sequence which has at least 80% identity with nucleotides 133 to 495 of SEQ ID NO: 15. Preferably, the nucleotide sequence has at least 85% identity, such as at least 90% identity, more preferably at least 95% identity, such as at least 96% identity, e.g. at least 97% identity, even more preferably at least 98% identity, such as at least 99% with nucleotides 133 to 495 of SEQ ID NO: 15. Preferably, the nucleotide sequence encodes a polypeptide having phospholipase activity.

The phospholipase may be derived from a strain of Fusarium, particularly F. venenatum, using probes designed on the basis of the DNA sequences in this specification. In one embodiment the phospholipase has phospholipase A activity.

The phospholipase may be produced by transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, such as a strain of Aspergillus, Fusarium, Trichoderma

or Saccharomyces, particularly A. niger, A. oryzae, F. venenatum, F. sambucinum, F. cerealis or S. cerevisiae, e.g. a glucoamylase-producing strain of A. niger such as those described in US 3677902 or a mutant thereof. The production of the phospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

The expression vector of the invention typically includes control sequences functioning as a promoter, a translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Sequence alignment and identity

Nucleotide sequences may be aligned with the AlignX application of the Vector NTI Program Suite 7.0 using the default settings, which employ a modified ClustalW algorithm (Thompson, J.D., Higgins, D.G., and Gibson T.J. (1994) Nuc. Acid Res. 22: 4673-4680), the swgapdnarnt score matrix, a gap opening penalty of 15 and a gap extension penalty of 6.66.

Amino acid sequences may be aligned with the AlignX application of the Vector NTI Program Suite v8 using default settings, which employ a modified ClustalW algorithm (Thompson, J.D., Higgins, D.G., and Gibson T.J., 1994), the blosum62mt2 score matrix, a gap opening penalty of 10 and a gap extension penalty of 0.1.

In one embodiment of the invention alignments of sequences and calculation of homology scores are done using the Lipman-Pearson Method (Lipman, D.J. and W.R. Pearson (1985) Rapid and sensitive protein similarity searches. Science 227: 1435-1441) using a PAM250 residue weight table (Dayhoff, M.O., R.M. Schwartz, and B.C. Orcutt (1978) A model of evolutionary change in proteins. In Dayhoff, M.O. (ed.), Atlas of Protein Sequence and Structure. National Biomedical Research Foundation. Washington, D.C. Vol 5. Suppl. 3: pp. 345-358) and the default settings of the MegAlign program, v4.03, in the Lasergene software package (DNASTAR Inc., 1228 South Park Street, Madison, Wisconsin 53715). The default settings are a K-tuple of 2, gap penalty of 4, and a gap length penalty of 12.

Phospholipid hydrolysis

The invention may be used in the hydrolysis of any phospholipid such as a lecithin, a cephalin or an inositide.

The invention may be used in analogy with prior art processes by replacing the phospholipase, e.g. in the production of baked products (WO 0032758, WO 9953769), mayonnaise (GB 1525929, US 4034124) or treatment of vegetable oil (US 5264367).

Use of phospholipase

The phospholipase of the invention can be used in various industrial application of phospholipases, e.g. as described below.

Use in baking

The phospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4567056 or WO 99/53769.

Use in detergent

The variant may be used as a detergent additive, e.g. at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per litre of wash liquor.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in GB 2247025, WO 9901531 or WO 9903962.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

Other uses

The phospholipase of the invention can be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution of slurry containing a starch hydrolyzate, especially a wheat starch hydrolyzate, since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Further, the phospholipase of the invention may be used for partial hydrolysis of phospholipids, preferably lecithin, to obtain improved phospholipid emulsifiers. This application is further described in Ullmann's Encyclopedia of Industrial Chemistry (Publisher: VCH

Weinheim (1996)), JP patent 2794574, and JP-B 6-087751.

Further, the phospholipase of the invention may be used in a process for the production of an animal feed which comprises mixing the phospholipase with feed substances and at least one phospholipid. This can be done in analogy with EP 743 017.

Even further the phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The phospholipase may e.g. be used in the processes described in JP-A 2-153997 and US 5264367.

Method for producing cheese

The phospholipase of the invention may be used for producing cheese in analogy with the process given in US 6399121.

In a preferred embodiment of the invention cheese is produced by contacting cheese milk or a fraction of cheese milk with a phospholipase of the invention and producing cheese from the cheese milk.

In a further preferred embodiment cheese is produced by contacting cheese milk or a fraction of cheese milk with a phospholipase, wherein the phospholipase comprises:

a) the sequence given by amino acids 146-153 of SEQ ID NO: 1, amino acids 87-94 of SEQ ID NO: 3, or amino acids 79-86 of SEQ ID NO: 12; or a sequence identical to any of these amino acid sequences except for the substitution of a single amino acid with another amino acid; and

b) two cysteine residues located on the N-terminal side of the sequence given in a); and

c) two cysteine residues located on the C-terminal side of the sequence given in a).

In the present context the term cheese milk is meant to cover any milk based composition used for production of cheese. A fraction of the cheese milk may be any fraction of the cheese milk such as e.g. cream, skim milk, milk, butter milk, butter or milk fat.

In a preferred embodiment cheese milk or a fraction of cheese milk is contacted with a phospholipase of the invention in an amount sufficient to decrease the oiling-off effect in cheese and/or to increase cheese yield. The oiling-off effect is the tendency of the cheese to form free oil upon storage and/or melting.

In one aspect the invention relates to a process for producing cheese comprising

treating a dairy composition with a phospholipase of the invention and producing cheese from the dairy composition.

Another aspect of the invention relates to a process for producing cheese comprising treating a dairy composition with phospholipase and producing cheese from the dairy composition, wherein the phospholipase is selected from the group of fungal/bacterial group XIII PLA2 phospholipases. In a preferred embodiment of the invention the fungal/bacterial group XIII PLA2 is from a fungus, more preferably from a fungus belonging to the *Ascomycetes*. A phospholipase belonging to the fungal/bacterial group XIII PLA2 may be any phospholipase belonging to this group as defined by Soragni et al., The EMBO Journal, 20 (2001), 5079-5090, and may e.g. be from the species *Tuber*, e.g. *T. borchii*, *Streptomyces*, e.g. *S. coelicor*, *Verticillium*, e.g. *V. dahliae*, *Aspergillus*, e.g. *A. oryzae*, *Neurospora*, e.g. *N. crassa*, or *Helicosporum*.

A dairy composition according to the invention may be any composition comprising milk constituents. Milk constituents may be any constituent of milk such as milk fat, milk protein, casein, whey protein, and lactose. A milk fraction may be any fraction of milk such as e.g. skim milk, butter milk, whey, cream, milk powder, whole milk powder, skim milk powder. In a preferred embodiment of the invention the dairy composition comprises milk, skim milk, butter milk, whole milk, whey, cream, or any combination thereof. In a more preferred embodiment the dairy composition consists of milk, such as skim milk, whole milk, cream, buttermilk, or any combination thereof.

The enzymatic treatment in the process of the invention may be conducted by dispersing the phospholipase into the dairy composition, and allowing the enzyme reaction to take place at an appropriate holding-time at an appropriate temperature. The treatment with phospholipase may be carried out at conditions chosen to suit the selected enzyme(s) according to principles well known in the art.

The enzymatic treatment may be conducted at any suitable pH, such as e.g., in the range 2-10, such as, at a pH of 4-9 or 5-7. In one embodiment the phospholipase treatment is conducted at 3-60°C, such as at 25-45°C (e.g., for at least 5 minutes, such as, e.g., for at least 10 minutes or at least 30 minutes, e.g., for 5-120 minutes). The phospholipase is added in a suitable amount to produce the cheese having the desired properties. Preferably, the phospholipase is added in an amount effective to decrease the oiling-off effect in cheese and/or to increase cheese yield. A suitable dosage of phospholipase will usually be in the range 0.001-0.5 mg enzyme protein per g milk fat, preferably 0.01-0.3 mg enzyme protein per g milk fat, more preferably, 0.02-0.1 mg enzyme protein per g milk fat

The cheeses produced by the process of the present invention comprise all varieties of cheese, such as, e.g. Campesino, Chester, Danbo, Drabant, Herregård, Manchego, Provolone, Saint Paulin, Soft cheese, Svecia, Taleggio, White cheese, including rennet-curd

cheese produced by rennet-coagulation of the cheese curd; ripened cheeses such as Cheddar, Colby, Edam, Muenster, Gruyere, Emmenthal, Camembert, Parmesan and Romano; blue cheese, such as Danish blue cheese; fresh cheeses such as Feta; acid coagulated cheeses such as cream cheese, Neufchatel, Quarg, Cottage Cheese and Queso Blanco. In a preferred embodiment the invention relates to a process for producing pasta filata cheese, such as e.g. Mozzarella and Pizza cheese. Pasta filata, or stretched curd, cheeses are normally distinguished by a unique plasticizing and kneading treatment of the fresh curd in hot water, which imparts the finished cheese its characteristic fibrous structure and melting and stretching properties, cf. e.g. "Mozzarella and Pizza cheese" by Paul S. Kindstedt, Cheese: Chemistry, physics and microbiology, Volume 2: Major Cheese groups, second edition, page 337-341, Chapman & Hall.

Sequence Listing and Deposited Microorganisms

The present application contains information in the form of a sequence listing, which is appended to the application and also submitted on a data carrier accompanying this application. In addition, the present application refers to deposited microorganisms. The contents of the data carrier and the deposited microorganisms are fully incorporated herein by reference.

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 B, D-38124 Braunschweig, Germany, and given the following accession number:

Deposit	Accession Number	Date of Deposit
E. coli	DSM 15441	12 February 2003
E. coli	DSM 15442	12 February 2003

MATERIALS AND METHODS

Media and substrates

Medium YP+2%G

10g yeast extract
20g peptone
water to 1L

autoclave at 121°C, 20 minutes
add 100ml 20% sterile glucose solution

RA sporulation medium:

50 g succinic acid
12.1 g sodium nitrate
1 g glucose
20 ml 50x Vogel's salts (Davis, R. H. and F. J. de Serres (1970), Meth. Enzymol. 17A:79-143)
components are blended in one liter distilled water and filter sterilized

Britton Robinson buffer

0.023 M phosphoric acid
0.023 M acetic acid
0.023 M boric acid
Titrated with NaOH or HCl to desired pH

Methods

Phospholipase activity (LEU)

Lecithin is hydrolyzed under constant pH and temperature, and the phospholipase activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

The substrate is soy lecithin (L-α-Phosphotidyl-Choline), and the conditions are pH 8.00, 40.0°C, reaction time 2 min. The unit is defined relative to a standard.

EXAMPLES

Example 1: Expression of a phospholipase A2 from *Tuber albidum* in *Aspergillus oryzae*

The DNA sequence disclosed in Soragni et al. (*supra*) was used to design primers for PCR amplification of TbSP1 from genomic DNA, with appropriate restriction sites added to the primer ends to facilitate cloning of the PCR product (SEQ ID NO: 13 and 14). A *Tuber albidum* strain, CBS 272.72, was obtained from the CBS (Centraalbureau voor Schimmelcultures, Utecht, The Netherlands), and cultured on X-agar at 20°C, as recommended by the CBS in List of Cultures, 1996. Mycelium was removed from the surface of the plate, and total DNA was isolated using a FastDNA Spin Kit (BIO101, Inc., Vista, CA), following the manufac-

turer's instructions. PCR amplification was performed using Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) following the manufacturers instructions and using an annealing temperature of 52°C for the first 5 cycles and 62°C for the last 25 cycles. A single PCR product was obtained, and the sequence was determined and is presented as SEQ ID 9 excluding the added synthetic restriction sites. Comparison of this genomic sequence to the cDNA sequence presented by E. Soragni et al. revealed a single intron. When the intron is removed, the nucleotide sequence from *T. albidum* CBS272.72 is 92.5% identical to that from *T. borchii* ATCC 96540, the strain used by E. Soragni et al. The corresponding peptide predicted from the *T. albidum* CBS272.72 gene sequence is 93.8% identical to the peptide sequence reported by Soragni et al.

The PCR fragment was restricted with *BamH*I and *Xho*I and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter, and has sequences for selection and propogation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the *amd*S gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (tpi) encoding-gene from *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The phospholipase A2-encoding gene of the resulting *Aspergillus* expression construct, pMStr70, was sequenced and the sequence was compared to that determined previously for the uncloned PCR fragment, SEQ ID 9. A single T to C mutation was found 52bp downstream of the stop codon.

Aspergillus oryzae was transformed with pMStr70 using standard techniques described in Christensen, T. et al., (1988), Biotechnology 6, 1419-1422. Transformants were cultured in YP+2%G medium shaken at 275 RPM at 30°C and expression of the Tuber phospholipase A2, TbPLA2, was monitored by SDS-PAGE.

Protein characterization

SDS-PAGE revealed two bands, with approximate Mw of 25 and16 kDa. The supernatant was purified by ion exchange chromatography on a SP-sepharose column equilibrated with 50 mM Acetate-buffer, and eluted with 1M NaCl pH 5.0. The two proteins eluted in two separate fractions. Protein concentration was determined using Protein Assay ESL from Roche. Activity was determined in the LEU assay.

	Mw kDa	Concentration mg/ml	Activity LEU/ml	Specific activity LEU/mg
Pool 1	23-25	1.32	61	46
Pool 2	16	0.42	272	648

The proteins were subjected to N-terminal sequencing. The N-terminal sequence of pool 1 (23-25 kDa band) was found to correspond to amino acids 32-50 of SEQ ID NO: 10. Blotting of pool 2 (16 kDa band) revealed two bands with N-terminal sequences corresponding to amino acids 86-98 and 91-103, respectively. Mass spectral analysis of the two bands showed masses of 13934 and 14348 Da respectively, matching within 5 Da of values calculated from the sequences of amino acids 86-210 and 91-210 of SEQ ID NO: 10, respectively.

Example 2: Purification procedure for two forms of *T. albidium* PLA2 expressed in *Aspergillus oryzae*.

In most fermentations of the *Aspergillus oryzae* transformant described in Example I that produces the *T. albidum* PLA2, two forms of the enzyme were detected during purification. One form ran at 22-23 kDa in SDS-PAGE and corresponds to the peptide reported by Soragni et al. (*supra*). Additionally, a new form was detected which ran at 16-17 kDa in SDS-PAGE and which has a high specific activity and a high isoelectric point.

Purification of the 22-23 kDa peptide

Fermentation supernatant containing phospholipase from *T.albidium* expressed in *A. oryzae* (prepared in Example 1) was sterile filtered using EKS filter purchased from Seitz Schenk Bad Kreuznach, Bettringerstrasse 42, Germany D-73550, Waldstetten.

The sterile filtered supernatant was then adjusted to pH of 8 and ionic strength under 4 mSi.

Anion Exchange chromatography

First step of purification was carried out on anion exchange chromatography using 50 ml Fast flow Q ™ sepharose column purchased from Amersham Pharmacia. The column was prequilibrated with 50 mM Tris acetate buffer pH 8. The sterile filtered fermentation broth was then applied on the column and the column was washed with the same buffer until all unbound material was washed out.

Bound proteins were eluted with the same buffer containing 1 M Sodium chloride pH

8 with flow rate of 5ml/minute and to a final volume of 500 ml total buffer. Fractions of 5ml each were collected using fraction collector and Phospholipase activity of all fractions containing was assayed qualitatively using Lecithin as substrate using L-α- Phosphatidyl choline purchased from Sigma product P-5638 and activity was assayed using NEFA C kit purchased from Wako Chemicals GmbH, Nissan Strasse 2, 41468 Neuss, Germany. Exact assay is described below.

Substrate solutions containing 10 mg/ml of Lecithin substrate were prepared in different buffers such as 50 mM Acetate pH 5 or 50 mM Hepes pH 7 or 50 mM Tris acetate pH 9 as buffers containing 2mM CaCl2 and 0.1 % Triton X-100 purchased from Fluka chemicals. Substrate was then emulsified by stirring and warming at 50°C and then cooling to 40°C and used as substrate.

Assay of activity was carried out using 300µl of the substrate emulsion incubated with 25 µl of the enzyme fractions for 20 minutes at 40°C then 30 µl of the assay mixture was transferred to 300 µl of the NEFA C color reagent A prepared as described by the manufacturer and incubated for 10 minutes at 37°C and 600 µl of the color reagent NEFA C B solution was added to the mixture and further incubated for 10 minutes. The blue color formed was then measured in a spectrophotometer at 505 nm.

Protein characterization

Fractions containing activity were then pooled and characterized for the molecular weight using SDS-PAGE electrophoresis using Novex Pre casted gels 4 to 20 % Tris–Glycine gels purchased from Invitrogen Life Technologies, Carlsbad CA 92008, USA.

22-23 kDa protein was detected and blotted and N-terminal analysis was carried out using an Applied Biosystem sequenator.

The first 19 amino acid residues from N-terminal were determined and found to have the sequence of amino acids 32-50 of SEQ ID NO: 10.

Purification of the the 16-17 kDa peptide

Sterile filtered fermentation supernatant of the *T. albidum* phospholiplase expressed in *A. oryzae* was adjusted to pH 4.7 and ionic strength was adjusted below 4 mSi.

Cation exchange chromatography

SP-sepharose ™ fast flow was purchased from Amersham Pharmacia. 50 ml Column was packed and equilibrated with 50 mM acetate buffer pH 4.7 the fermentation supernatant was then applied on column and unbound material was washed using the same buffer.

Bound protein with high pI was then eluted with a linear salt gradient using 50 mM acetate buffer pH 4.7 containing 1 M Sodium chloride. Fractions and flow rate were similar to those used for the low pI form of the phospholipase. Phospholipase activity in the fractions was assayed qualitatively using NEFA kit as above. Fractions containing Phospholipase ac-

tivity were pooled and SDS-PAGE was carried out as described above.

16-17 kDa protein was observed which had a high isoelectric point, above 9.

The N-terminal analysis of the protein was carried out after blotting the protein and using Applied biosystem sequentaor which showed an N-terminal which was completely different from the one published in Soragni et al. (*supra*). Thus, the *T albidum* PLA2 was found to have two forms deriving from differential N-terminal processing with N-terminal sequences corresponding to amino acids 86-105 and 91-110 of SEQ ID NO: 10, respectively.

Example 3: Cheese making with *T. albidum* phospholipase

Pasteurized, non-homogenized cream (North Carolina State University Dairy Plant) was used to standardize five hundred grams pasteurized, non-homogenized skim milk (North Carolina State University Dairy Plant) to 3.5% fat thus producing full fat mozzarella cheese. The cheese milk for each experiment was treated with either the 16-17 kD *T. albidum* phospholipase prepared according to example 2, or the commercial phospholipase Lecitase® 10L (Novozymes A/S, Bagsværd, Denmark), and placed in a 35°C water bath until equilibrated to that temperature. The initial pH of the cheese milk was taken and 0.01% (w/w) of starter culture was added.

pH was monitored until a pH of 6.4 was reached. 250 µl rennet (Novozym 89L) was diluted to in 9 ml total solution with deionised water, one ml of this solution was added to the cheese milk and the cheese milk was stirred vigorously for 3 minutes. The stir bar was removed and the rennetted milk was allowed to sit at 35°C.

After the above treatments, curd was ready to cut when a spatula was inserted and sharp edges were seen. The cheese was cut by pushing the cutter down and while holding the beaker quickly turning the cutter and finally pulling the cutter up. The curd was allowed to rest 5 minutes then stirred gently with spoon. Temperature was raised to 41°C with intermittent gentle agitation for ~ 45 min or until the pH dropped to 6.0-5.9. The curd was drained using cheesecloth then replaced in the beaker and kept at 41°C in water bath while pouring off whey as needed.

When the curd reached pH 5.3, the stainless steel bowl with the curd in it was flooded in a water bath at 69°C for 5 minutes then hand stretched. Curd was tempered in cold icewater for 30 minutes. The cheese curd was dried out with paper towel, weighed and refrigerated overnight.

Control cheese making experiments were made from the same batch of milk following the same procedures except that no phospholipase was added.

Actual cheese yield was calculated as the weight of cheese after stretching relative to the total weight of cheese milk.

Moisture adjusted cheese yield was expressed as the actual yield adjusted to standard constant level of moisture. Moisture adjusted yield was calculated by multiplying the actual yield and the ratio of actual moisture content to standard moisture, according to the following formula:

$$Y_{adj} = (Y_{act} \times 1 - M_{act}) / (1 - M_{std})$$

where Y_{adj} = moisture adjusted cheese yield, Y_{act} = actual cheese yield, M_{act} = actual moisture fraction & M_{std} = standard moisture fraction (0.48).

The moisture adjusted cheese yield of all experiments and controls are shown in table 1

Table 1

Treatment	Phospholipase mg enzyme protein/g fat	Moisture adjusted cheese yield	Yield increase compared to control
Control	0	10.72	
T. albidum PLA2	0.055	11.04	2.9%
Control	0	11.25	
T. albidum PLA2	0.055	11.57	2.8%
Control	0	9.22	
Lecitase® 10L	0.18	9.48	2.7%
Control	0	9.62	
Lecitase® 10L	0.18	9.90	2.8%

Example 4: Cloning and expression of a phospholipase (FvPLA2) from *Fusarium venenatum* in *Aspergillus oryzae*

Cells of the *Fusarium venenatum* A3/5 (originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, Fungal Genetics and Biology 23: 62-80; and O'Donnell et al., 1998, Fungal Genetics and Biology 23: 57-67) were grown for two days in Vogel's minimal medium (Davis, R. H. and F. J. de Serres (1970), Meth. Enzymol. 17A:79-143) at 28°C in shaking culture, filtered on sterile Miracloth (Calbiochem, San Diego, California, USA), and transferred to

"RA sporulation medium" in which they were incubated in shaking culture for an additional 24 hr at 28°C. Cells and spores were collected by centrifugation and lysed, and RNA was extracted and transcribed into cDNA that was cloned into pZErO-2 by the methods described in WO 00/56762. The number of independent clones in this library before amplification was 2.5x105, of which 92% contained inserts ranging in size from 550-2500 bp. Partial DNA sequences were determined for approximately 1000 randomly chosen clones and the sequences were stored in a computer database by methods described in WO 00/56762.

The nucleotide sequence of a cDNA encoding TbSP1, a phospholipase A2 from *Tuber borchii*, and the corresponding peptide translation were reported by E. Soragni et al., 2001. This translated peptide sequence was compared to translations of the *Fusarium venenatum* partial cDNA sequences using the TFASTXY program, version 3.3t08 (Pearson et al., 1997). One translated *F. venenatum* sequence was identified as having 42% identity to TbSP1 through a 125 amino acid overlap. The complete sequence of the cDNA insert of the corresponding clone, FM0700, was determined and is presented as SEQ ID NO: 15, and the peptide translated from this sequence, FvPLA2, is presented as SEQ ID NO: 16. This sequence was used to design the primers FvPLA1 and FvPLA2.2 for PCR amplification of the FvPLA2 encoding-gene from FM0700, with appropriate restriction sites added to the primer ends to facilitate sub-cloning of the PCR product.

FvPLA1: CTGGGATCCTCAAGATGAAGTTCAGCG

FvPLA2.2: GACCTCGAGACCCGCCATTTAAGATT

PCR amplification was performed using Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) following the manufacturers instructions and using an annealing temperature of 52°C and an extension temperature of 60°C for 20 cycles.

The PCR fragment was restricted with BamHI and XhoI and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the amdS gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (tpi) encoding-gene from *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The phospholipase-encoding gene of the resulting *Aspergillus* expression construct, pMStr77, was sequenced and the sequence agreed completely with that determined previously for the insert of FM0700.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr77

using standard techniques (T. Christensen et al., 1988). Transformants were cultured in YP+2%G medium shaken at 275 RPM at 30°C and expression of FvPLA2 was monitored by SDS-PAGE.

A strain of *Eschericia coli* containing a gene encoding the phospholipase from *F. venenatum* was deposited by the inventors under the terms of the Budapest Treaty with Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany. The deposit date was 12 February 2003, and the accession number was DSM 15442.

Example 5: Purification and sequence comparison of FvPLA2

FvPLA2 from the fermentation of example 4 was purified by ion exchange chromatography on a SP-sepharose column equilibrated with 50 mM Acetate-buffer pH 4.7, and eluted with 1M NaCl pH 4.7. Fractions were analyzed on SDS-PAGE, and fractions containing a 14 kDa protein were pooled. The identity of the pure protein was confirmed by determining the the N-terminal sequence, which was identical to the sequence from amino acid (aa) 29-40 of SEQ ID NO: 16. Additionally, the mass of the peptide was determined by mass spectral analysis, because the apparent size estimated from SDS-PAGE, 14 kDa, is smaller than that of the peptide predicted by processing the theoretical peptide in SEQ ID NO: 16. The mass of the purified, active FvPLA2 was found to be 13336 Da. This molecular mass indicates additional processing at the C-terminus, and is consistent with a cleavage between amino acids 149 and 150 in SEQ ID NO: 16, as the peptide sequence from amino acid 29 to 149 has a theoretical mass of 13335,66 Da.

A comparison of the mature processed peptide (amino acids 29-149 of SEQ ID NO: 16) with known sequences showed that the closest prior-art sequence was a phospholipase from *Verticillium dahliae* translated from Unisequence ID: VD0100C34 from the COGEME Phytopathogenic Fungi and Oomycete EST Database Version 1.2 (http://cogeme.ex.ac.uk/) (Soanes et al. (2002) Genomics of phytopathogenic fungi and the development of bioinformatic resources. Mol Plant Microbe Interact. 15(5):421-7). The processing of the partial peptide predicted from the *V. dahliae* sequence was estimated by comparison to the found processing for FvPLA2. The identity between amino acids 29 to 149 of SEQ ID NO: 16 and the estimated sequence of the mature peptide of the *V. dahliae* phospholipase was calculated to be 77%.

Example 6: Physical properties of FvPLA2

Catalytic activity

Phospholipase activity as a function of enzyme concentration was determined in the LEU assay for FvPLA2 of example 4. Results are shown in table 1.

Table 1

Enzyme conc. (µg/ml)	LEU (µeq NaOH/min)
71.1	14.0
53.3	12.7
21.3	10.6
10.7	7.4
5.3	5.6
2.7	4.1

Temperature profile

The enzyme activity as a function of temperature was determined for an enzyme solution with a concentration of 5.3 µg/ml. Other conditions as in the LEU assay. Results are shown in table 2.

Table 2

Temperature (°C)	LEU (µeq NaOH/min)
25	3.10
35	4.87
40	5.41
45	6.97
50	7.86
55	9.03
60	8.27
65	6.90

pH stability

The enzyme was diluted in a Britton Robinson buffer at the specified pH for 30 min at 30°C. After further dilution in water catalytic activity was measured in the LEU assay. Results

are shown in table 3.

Table 3

pH	LEU (µeq NaOH/min)
2	3.78
3	5.11
4	5.60
5	5.49
6	5.37
7	5.61
8	5.52
9	5.64
10	5.50
11	5.21

Thermo stability

The enzyme was diluted in Britton Robinson buffer at pH 3 and 10 respectively, and at pH 7 with 30% sorbitol. After incubation at the specified temperature for 30 minutes, the solution was cooled to the reaction temperature and assayed in the LEU assay. The results are shown in table 4; activities are given relative to the highest measured activity.

Table 4. Relative activity (%) as a function of pH and temperature

Temperature (°C)	pH 3	pH 10	pH7/30% sorbitol
30	100%	100%	87%
40	95%	92%	100%
50	16%	14%	68%
60	1%	0%	2%

Example 7: Cheese making with FvPLA2

Pasteurized, non-homogenized cream (North Carolina State University Dairy Plant) was used to standardize five hundred grams pasteurized, non-homogenized skim milk (North Carolina State University Dairy Plant) to 3.5% fat thus producing full fat mozzarella cheese.

The cheese milk for each experiment was treated with either the *F. venenatum* phospholipase (FvPLA2) prepared according to example 5, or of the commercial phospholi-

pase Lecitase® 10L (Novozymes A/S, Bagsværd, Denmark), and placed in a 35°C water bath until equilibrated to that temperature. The initial pH of the cheese milk was taken and 0.01% (w/w) of starter culture at was added.

pH was monitored until a pH of 6.4 was reached. 250 µl rennet (Novozym 89L) was diluted to in 9 ml total solution with deionized water, one ml of this solution was added to the cheese milk and the cheese milk was stirred vigorously for 3 minutes. The stir bar was removed and the rennetted milk was allowed to sit at 35°C.

After the above treatments, curd was ready to cut when a spatula was inserted and sharp edges were seen. The cheese was cut by pushing the cutter down and while holding the beaker quickly turning the cutter and finally pulling the cutter up. The curd was allowed to rest 5 minutes then stirred gently with spoon. Temperature was raised to 41°C with intermittent gentle agitation for ~ 45 min or until the pH dropped to 6.0-5.9. The curd was drained using cheesecloth then replaced in the beaker and kept at 41°C in water bath while pouring off whey as needed.

When the curd reached pH 5.3, the stainless steel bowl with the curd in it was flooded in a water bath at 69°C for 5 minutes then hand stretched. Curd was tempered in cold icewater for 30 minutes. The cheese curd was dried out with paper towel, weighed and refrigerated overnight.

Control cheese making experiments were made from the same batch of milk following the same procedures except that no phospholipase was added.

Actual cheese yield was calculated as the weight of cheese after stretching relative to the total weight of cheese milk.

Moisture adjusted cheese yield was expressed as the actual yield adjusted to standard constant level of moisture. Moisture adjusted yield was calculated by multiplying the actual yield and the ratio of actual moisture content to standard moisture, according to the following formula:

$$Yadj = Yact \times (1- Mact) / (1-Mstd)$$

where Yadj = moisture adjusted cheese yield, Yact = actual cheese yield, Mact = actual moisture fraction & Mstd = standard moisture fraction (0.48).

The moisture adjusted cheese yield of all experiments and controls are shown in table 5.

Table 5

Treatment	Phospholipase mg enzyme protein/g fat	Moisture adjusted cheese yield	Yield increase compared to control
Control	0	11.70	
FvPLA2	0.071	11.95	2.1%
Control	0	11.50	
FvPLA2	0.071	11.83	2.8%
Control	0	9.22	
Lecitase® 10L	0.18	9.48	2.7%
Control	0	9.62	
Lecitase® 10L	0.18	9.90	2.8%

Example 8: Cheese making with FvPLA2

Milk was pasteurized at 72°C for 15 seconds and then cooled to below 10°C. Milk was standardized to 2.4% fat with cream. After standardization the milk was preheated in a heat exchanger at a pre-ripening temperature of 34.5°C. 150 kg milk was poured into each cheese vat and 15 g culture (F-DVS ST-M6) was added. The phospholipase from example 5 was added in a dosage of 5 LEU/g fat and the milk was incubated for 1 h at 34.5°C. Rennet (Chy-Max Plus, 200 IMCU) was added and agitation was continued for not more than 4 min.

After approx. 60 min when the coagulum was judged ready it was cut using 10 mm knives. The agitator was returned to the vat and after 10 min. the scalding was started by increasing the temperature to 41°C within 30 min. After reaching 41°C a further stirring for approximately 20 min. took place until a titratable acidity of 0.15-0.16% was reached. The curd was allowed to settle in the vat, and whey was drained. The curd was cut in uniform blocks and the blocks were turned and stacked into two. Subsequently, at intervals of 10 min. the blocks were turned and kept in stacks of two. At a pH of around 5.15-5.20, the curd was milled in a milling machine. The curd pieces were added two percent of salt (weight/weight).

After milling all the curd was added into the stretcher, which contains 70 l preheated water at 74°C. Around 20 l of hot water was transferred to the upper chamber and the cheese is added. When the curd temperature reached 62°C, the stretching was stopped and the curd moved to the extruder. Cheeses were extruded into 8-9 cheese loaves, each of 2.3 kg, and cooled in 5-7°C water for 20 min. After 20 min. cooling the cheeses were moved to the saturated brine and brined for 1.5 hours at 5-6°C. The brine was made by blending 120 kg water, adding salt to 22Be, 750 g CaCl2 (34% solution) and adjusted to pH 5.1. After brining each cheese was dried for around 30 min. and weighed before vacuum packaging. Samples were taken for pH and compositional analyses (moisture, salt, fat and protein) after about 1 week's storage in cold room.

Actual yield (AY) was adjusted to 48% moisture in cheese:

$$\textbf{Adj Yield} = \frac{AY \times (100 - \%\ \text{moisture})}{100 - 48}$$

Table 6

	Adjusted yield (kg) Control	Adjusted yield (kg) Experimental	Average yield increase (kg)	Yield increase (%)
Day 1	10.62	10.81		
	10.70	10.90	0.195	1.8
Day 2	9.90	10.16		
	9.95	10.14	0.225	2.3
Day 3	10.00	10.15		
	10.01	10.16	0.15	1.5

Example 9: Over-expression of *Aspergillus oryzae* PLA2 (AoPLA2) in *Aspergillus oryzae*

Medium

DAP2C-1

11g $MgSO_4 \cdot 7H_2O$

1g KH_2PO_4

2g Citric acid, monohydrate

30g maltodextrin

6g $K_3PO_4 \cdot 3H_2O$

0.5g yeast extract

0.5ml trace metals solution

1ml Pluronic PE 6100 (BASF, Ludwigshafen, Germany)

Components are blended in one liter distilled water and portioned out to flasks, adding 250 mg CaCO3 to each 150ml portion.

The medium is sterilized in an autoclave. After cooling the following is added to 1 liter of medium:

23 ml 50% w/v $(NH_4)_2HPO_4$, filter sterilized

33 ml 20% lactic acid, filter sterilized

Trace metals solution

6.8g $ZnCl_2$

2.5g $CuSO_4 \cdot 5H_2O$

0.24g $NiCl_2 \cdot 6H_2O$

13.9g $FeSO_4 \cdot 7H_2O$

8.45g $MnSO_4 \cdot H_2O$

3g Citric acid, monohydrate

Components are blended in one liter distilled water.

The cloning and partial sequencing of a cDNA encoding a phospholipase A2 from *Aspergillus oryzae* is described in WO 00/56762. The full sequence of the clone, AS3812, is given in SEQ ID NO: 6.

This sequence was used to design the primer AoPLA1 for use with the vector primer pYESrev in PCR amplification of the PLA2 encoding-gene from AS3812 with the addition of a restriction site to facilitate sub-cloning of the PCR product:

AoPLA1: TGAGGATCCATCATGAAGAACATCTTCG

pYESrev: gggcgtgaatgtaagcgtgac

PCR amplification was accomplished using Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) following the manufacturers instructions and using an annealing temperature of 52°C for the first 5 cycles and 62°C for the last 25 cycles, and an extension time of 1.5 minutes.

The PCR fragment was restricted with BamHI and XhoI and cloned into the *Aspergillus* expression vector pMStr57 (described in Example 1) using standard techniques. The phospholipase-encoding gene of the resulting *Aspergillus* expression construct, pMStr71, was sequenced and the sequence agreed completely with that determined previously for the insert of AS3812.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr71 using standard techniques (T. Christensen et al., 1988). Transformants were cultured in DAP2C-1 medium shaken at 270 RPM at 37°C for 4 days and expression of phospholipase was monitored by SDS-PAGE.

Example 10: Purification and determination of peptide processing

The *Aspergillus oryzae* phospholipase from the fermentation of example 9 was filtered through 0.22μ sterile filter Seitz-EKS obtained from Pall Corporation (Pall SeitzSchenk Filter Systems GmbH Pianiger Str.137 D-55543 Bad Kreuznach, Germany). The sterile filtered solution was then adjusted to pH 4.7 using dilute acetic acid. Ionic strength of the fermentation supernatant was then adjusted so that salt concentration was low and ionic strength was under 4 mSi. Purification of the desired PLA2 protein was obtained by cation exchange chromatography using SP sepharose fast Flow matrix obtained from Amersham-Pharmacia (Sweden). The cation exchanger matrix was packed washed and pre-equilibrated with 50 mM Sodium acetate buffer pH 4.7 (Buffer A) on XK26 column obtained from Amersham Pharmacia. Fermentation supernatant containing the desired PLA2 adjusted for pH and ionic strength was then applied on the column. Unbound material was then washed with the buffer A until all the UV absorbing material was washed out, which was monitored by UV detector attached to fraction collector equipment. Bound proteins were then eluted with a linear salt gradient using Buffer B, which contained 1 M Sodium chloride as salt in 50 mM Sodium acetate buffer pH 4.7. Total volume of the linear gradient reaching 1 M salt concentration was around 500 ml (10 column volume). Fractions of 10 ml each were collected during the elution. All the fractions were assayed for phospholipase activity using Lecithin as substrate obtained from Sigma chemicals. Fatty acids released from Lecithin on incubation with the phospholipase were detected using NEFA C kit obtained from Waco chemicals. Fractions containing phospholipase activity were then checked for purity of the protein using standard SDS-PAGE technique. Fractions were pooled that contained a single band of the desired PLA2 showing molecular weight of around 16 kDa, as determined by comparison to molecular weight standards from Amersham-Pharmacia.

The identity of the pure protein was confirmed by determining the N-terminal sequence, which was identical to the sequence from amino acid (aa) 37-45 of SEQ ID NO: 7. Additionally, the mass of the peptide was determined by mass spectral analysis. The purified,

active *Aspergillus* PLA2 gave two masses, 14114 and 14242 Da. These molecular masses indicate additional processing at the C-terminus, consistent with cleavage between amino acids 121 and 122 in SEQ ID NO: 7, as the peptide sequence from amino acid 37 to 121 has a theoretical mass of 14114.11 Da and cleavage between amino acids 122 and 123, predicting the peptide sequence from amino acid 37 to 123 with a theoretical mass of 14242.29 Da.

Example 11: Expression of incompletely processed phospholipase from *Aspergillus oryzae* and *Fusarium venenatum*

Processing of the *Aspergillus oryzae* PLA2 (AoPLA2) and the *Fusarium venenatum* PLA (FvPLA2) at both the N- and C-termini occurs at single or multiple basic residues (lys or arg), typical of the cleavage sites of the Kexin-like maturases, which are often responsible for processing propeptides (Jalving, R., et al. (2000) Appl. Environ. Microbiol. 66: 363-368). In order to determine the effect of processing on the activity of AoPLA2 and FvPLA2, the enzymes were expressed in a Kexin deficient strain of *Aspergillus oryzae*. Processing was then assessed by SDS-PAGE, and phospholipase activity was measured for cultures of strains expressing AoPLA2 and FvPLA2 in both wild-type and Kexin deficient backgrounds.

A Kexin deficient strain of *Aspergillus oryzae* (*kex*B$^-$) was constructed by a disrupting the *kex*B gene of *A. oryzae* (EMBL:AB056727) by methods established in the art, such as those described in WO 98/12300 and US6013452. Disruption of *kex*B was confirmed by Southern blot analysis and by monitoring the expression of peptides where KexB is known to be responsible for maturation. The *kex*B$^-$ strain was transformed with the AoPLA2 expression construct described in Example 9, and with the FvPLA2 expression construct described in Example 4. These strains were fermented in YP+2%G at 30°C, along with the *kex*B$^+$ expression strains for both AoPLA2 and FvPLA2 described in Examples 9 and 4, and untransformed strains as controls. AoPLA2 expressing strains were shaken at 200RPM for 4 days while FvPLA2 expressing strains were shaken at 275RPm for 3 days. Phospholipase expression and processing were assessed by SDS-PAGE.

In SDS-PAGE analysis, AoPLA2 was resolved as a distinct single band in both *kex*B$^+$ and *kex*B$^-$ strains. When expressed in the *kex*B$^+$ strain, AoPLA2 ran at ca. 16 kDa, consistent with the migration observed earlier for fully processed AoPLA2 (Example 10), while in the *kex*B$^-$ strain, AoPLA2 ran at ca. 27-28 kDa, consistent with a lack of processing or incomplete processing. When expressed in the *kex*B$^+$ strain FvPLA2 was resolved as two bands with apparent molecular weights of 17kDa and 14kDa. The 14kDa band corresponds to the fully processed peptide (Example 5), while the 17kDa peptide is a partially processed form.

When expressed in the *kex*B$^-$ strain, FvPLA2 ran as a single band at ca. 18-19kDa, a size consistent with incomplete processing. No similar bands were seen in any of the control samples from untransformed strains. Relative band intensities suggest that expression of AoPLA2 in the *kex*B$^-$ strain was 1/5 to 1/10 the level of that in the *kex*B$^+$ strain, while expression of FvPLA2 in the *kex*B$^-$ strain was the same to 1/2 the level of that in the *kex*B$^+$ strain.

The activity of the phospholipases produced by each strain was determined in the LEU assay and is shown in table 7.

Table 7

Strain genotype			Activity LEU/ml
KexB	FvPLA	AoPLA2	
+	-	-	0
-	-	-	0
+	+	-	38
-	+	-	0
+	-	+	56
-	-	+	0

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0-2	International Application No.	
0-3	Applicant's or agent's file reference	10342.504-WO

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1-2	line	24
1-3	Identification of deposit	
1-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	12 February 2003 (12.02.2003)
1-3-4	Accession Number	DSMZ 15441
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
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2-3	Identification of deposit	
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2-3-3	Date of deposit	12 February 2003 (12.02.2003)
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2-5	Designated States for Which Indications are Made	all designations

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0-4-1	Authorized officer	

FOR INTERNATIONAL BUREAU USE ONLY

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CLAIMS

1. A method of producing a phospholipase which comprises processing an expressed fungal peptide so as to cleave off a peptide from the C-terminal end and/or a peptide from the N-terminal end to obtain a core peptide with phospholipase activity, wherein the core peptide comprises

a) the amino acid sequence given by amino acids 146-153 of SEQ ID NO: 1, amino acids 87-94 of SEQ ID NO: 3, or amino acids 79-86 of SEQ ID NO: 12; or a sequence identical to any of these amino acid sequences except for the substitution of a single amino acid with another amino acid; and

b) at least two cysteine residues located on the N-terminal side of the sequence given in a); and

c) at least two cysteine residues located on the C-terminal side of the sequence given in a).

2. The method of claim 1 wherein the expressed peptide is expressed in a filamentous fungal host cell transformed with DNA encoding the expressed peptide.

3. The method of claim 2 wherein the host cell is an *Aspergillus*, *Fusarium* or *Trichoderma*, particularly *A. oryzae*, *A. niger*, *F. venenatum* or *T. reesei*.

4. The method of claim 2 wherein the expressed peptide is processed *in vivo* by the host cell.

5. The method of any of the claims 1-4 wherein the core peptide has a length of 100-150 amino acids.

6. The method of any of the claims 1-5 wherein the phospholipase has a specific phospholipase activity which is at least 2 times the activity of the expressed peptide before processing.

7. The method of any of the claims 1-6 wherein the expressed peptide is derived from *Tuber*, *Verticillium*, *Neurospora*, *Aspergillus*, or *Helicosporum*, particularly *T. borchii*, *T. albidum*, *V. dahliae*, *V. tenerum*, *N. crassa*, *A. oryzae*, or *Helicosporium* sp.HN1.

8. The method of any of the claims 1-7 wherein the expressed peptide is cleaved within 0-18 amino acids on the N-terminal side of the sequence aligning with amino acids 97-101 of SEQ ID NO: 1, when the complete expressed peptide sequence is aligned simultaneously with the sequences given in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID

NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

9. The method of any of the claims 1-8 wherein the expressed peptide is cleaved within 0-11 amino acids on the C-terminal side of the sequence aligning with amino acids 204-209 of SEQ ID NO: 1, when the complete expressed peptide sequence is aligned simultaneously with the sequences given in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

10. The method of any of claims 1-9 wherein the expressed peptide is cleaved within 10 amino acids of a Kex2 processing site or within 11 amino acids of an FG sequence or both.

11. A method of hydrolyzing a phospholipid comprising contacting the phospholipid with a phospholipase produced by the method of any of the claims 1-10.

12. A method of producing cheese comprising contacting cheese milk or a fraction of cheese milk with a phospholipase, wherein the phospholipase comprises:

a) the sequence given by amino acids 146-153 of SEQ ID NO: 1, amino acids 87-94 of SEQ ID NO: 3, or amino acids 79-86 of SEQ ID NO: 12; or a sequence identical to any of these amino acid sequences except for the substitution of a single amino acid with another amino acid; and

b) two cysteine residues located on the N-terminal side of the sequence given in a); and

c) two cysteine residues located on the C-terminal side of the sequence given in a).

13. A method of producing cheese comprising contacting cheese milk or a fraction of cheese milk with a phospholipase produced by the method of any of the claims 1-10.

14. A phospholipase which is a polypeptide having an amino acid sequence which is at least 80 % identical with amino acids 91-210 in SEQ ID NO: 10 (*T. albidum*), amino acids 92-211 in SEQ ID NO: 1 (*T. borchii*), amino acids 30-137 in SEQ ID NO: 12 (*V. tenerum*), amino acids 38-145 in SEQ ID NO: 3 (*V. dahliae),* amino acids 44-151 in SEQ ID NO: 4 (*N. crassa*), amino acids 37-157 in SEQ ID NO: 7 (*A. oryzae*), or amino acids 58-168 in SEQ ID NO: 8 (*N. crassa*).

15. A phospholipase which comprises:

a) a polypeptide encoded by the phospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 15442; or

b) a polypeptide comprising the amino acid sequence of amino acids 29 to 149 of SEQ ID NO: 16, or an amino acid sequence which can be obtained therefrom by

substitution, deletion, and/or insertion of one or more amino acids;

or

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 80 % homology with said polypeptide, or

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form, or

iii) is an allelic variant of said polypeptide;

or

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with a complementary strand of the nucleic acid sequence of nucleic acids 133 to 495 SEQ ID NO: 15 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

16. The phospholipase of claim 15 which is native to a strain of *Fusarium*, particularly *F. venenatum*.

17. A nucleic acid sequence comprising a nucleic acid sequence which encodes the phospholipase of claims 15 or 16.

18. A nucleic acid sequence which comprises:

a) the partial DNA sequence encoding a mature phospholipase cloned into a plasmid present in *Escherichia coli* DSM 15442,

b) the partial DNA sequence encoding a mature phospholipase of nucleic acids 133 to 495 of SEQ ID NO: 15,

c) an analogue of the sequence defined in a) or b) which encodes a phospholipase and

i) has at least 80 % homology with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

19. A nucleic acid construct comprising the nucleic acid sequence of claims 17 or 18 operably linked to one or more control sequences capable of directing the expression of the phospholipase in a suitable expression host.

20. A recombinant expression vector comprising the nucleic acid construct of claim 19, a promoter, and transcriptional and translational stop signals.

21. A recombinant host cell comprising the nucleic acid construct of claim 20.

22. A method for producing a phospholipase comprising cultivating the host cell of claim 21 under conditions conducive to production of the phospholipase, and recovering the phospholipase.

23. A method for preparing a dough or a baked product made from the dough, comprising adding the phospholipase of claim 15 to the dough.

24. A dough composition comprising the phospholipase of claim 15.

25. A detergent composition comprising a surfactant and the phospholipase of claim 15.

26. A process for reducing the content of phosphorus in a vegetable oil, comprising contacting the oil with the phospholipase of claim 15 in the presence of water, and then separating an aqueous phase from the oil.

27. A process for producing cheese comprising treating a dairy composition with a phospholipase and producing cheese from the dairy composition, wherein the phospholipase is the phospholipase of claim 15.

28. A process for producing cheese comprising treating a dairy composition with a phospholipase and producing cheese from the dairy composition, wherein the phospholipase is selected from the group of fungal/bacterial group XIII PLA2 phospholipases.

01 SQ listing ST25 23-APR-2004
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Ser Asp Val Phe Asp Gly Met Asp Ala Asp Glu Ala Arg Asp Ala Leu
195 200 205

Asn Pro Tyr Leu Ser Glu Glu Lys Thr Lys Glu Tyr Tyr Asp Arg Ala
210 215 220

Leu Ala Arg Tyr Asn Lys Cys Val Glu Glu Ala Met Ala Gln Gly Ile
225 230 235 240

Asp Leu Gln Lys Tyr Trp Ala Ala Phe
245

<210> 9
<211> 832
<212> DNA
<213> Tuber albidum

<220>
<221> CDS
<222> (2)..(426)

<220>
<221> CDS
<222> (476)..(680)

<400> 9

a atg gtc aag att gct gcc att gtc ctc cta atg gga att cta gcc aat 49
Met Val Lys Ile Ala Ala Ile Val Leu Leu Met Gly Ile Leu Ala Asn
1 5 10 15

gct gcc gcc atc cct gtc agc gag cca gca gcc ctg gcg aag cgt gga 97
Ala Ala Ala Ile Pro Val Ser Glu Pro Ala Ala Leu Ala Lys Arg Gly
20 25 30

aac gct gag gtc att gct gaa caa act ggt gat gtc ccg gat ttc aac 145
Asn Ala Glu Val Ile Ala Glu Gln Thr Gly Asp Val Pro Asp Phe Asn
35 40 45

act caa att aca gag cca act ggg gag gga gac cgt ggg gat gtg gtc 193
Thr Gln Ile Thr Glu Pro Thr Gly Glu Gly Asp Arg Gly Asp Val Val
50 55 60

gac gaa acc gat ttg tcc acg gat att gtc cca gag acc gag gct gct 241
Asp Glu Thr Asp Leu Ser Thr Asp Ile Val Pro Glu Thr Glu Ala Ala
65 70 75 80

tcc ttc gcc gct agt tca gta tct gca gcc tca cca gca tct gac acc 289
Ser Phe Ala Ala Ser Ser Val Ser Ala Ala Ser Pro Ala Ser Asp Thr

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                85                  90                  95

gac agg ctt ctc tac tca acc tcc atg ccc gcc ttc ttg act gct aag      337
Asp Arg Leu Leu Tyr Ser Thr Ser Met Pro Ala Phe Leu Thr Ala Lys
            100                 105                 110

cgc aat aag aac ccc ggc aac ttg gac tgg agc gat gat gga tgc agc      385
Arg Asn Lys Asn Pro Gly Asn Leu Asp Trp Ser Asp Asp Gly Cys Ser
        115                 120                 125

aac tcc ccg gac agg cct gca ggg ttt aac ttc ctt gac tc               426
Asn Ser Pro Asp Arg Pro Ala Gly Phe Asn Phe Leu Asp Ser
    130                 135                 140

gtaagtcctc cttcatttat gctatctaca ttcactaata ttcgaacag c tgc aag      482
                                                    Cys Lys


cgt cac gac ttc ggg tac cgc aac tac aag aag cag cgc cgc ttc aca      530
Arg His Asp Phe Gly Tyr Arg Asn Tyr Lys Lys Gln Arg Arg Phe Thr
145                 150                 155                 160

gag cct aat cgc aag cgc att gat gac aat ttc aag aag gac cta tat      578
Glu Pro Asn Arg Lys Arg Ile Asp Asp Asn Phe Lys Lys Asp Leu Tyr
                165                 170                 175

aat gag tgc gcc aag tac tct ggc ctc caa tcc tgg aaa ggt gtt gcc      626
Asn Glu Cys Ala Lys Tyr Ser Gly Leu Gln Ser Trp Lys Gly Val Ala
            180                 185                 190

tgc cgc aaa atc gcg aac act tac tac gat gct gta cgc tcc ttc ggt      674
Cys Arg Lys Ile Ala Asn Thr Tyr Tyr Asp Ala Val Arg Ser Phe Gly
        195                 200                 205

tgg ttg taaatgtgcg gaagagatat caagtgggat cgaggaagag gatggtgaaa      730
Trp Leu
    210

gagctgagag gtggatttct ttacattccg caatggctac tacagaagaa ctgtgctcct  790

caaatttaat ctcatttttg tgtctatcta tccactctag aa                     832


<210>  10
<211>  210
<212>  PRT
<213>  Tuber albidum

<400>  10

Met Val Lys Ile Ala Ala Ile Val Leu Leu Met Gly Ile Leu Ala Asn
1               5                   10                  15


Ala Ala Ala Ile Pro Val Ser Glu Pro Ala Ala Leu Ala Lys Arg Gly
            20                  25                  30


Asn Ala Glu Val Ile Ala Glu Gln Thr Gly Asp Val Pro Asp Phe Asn
        35                  40                  45


Thr Gln Ile Thr Glu Pro Thr Gly Glu Gly Asp Arg Gly Asp Val Val
    50                  55                  60


Asp Glu Thr Asp Leu Ser Thr Asp Ile Val Pro Glu Thr Glu Ala Ala
65                  70                  75                  80
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Ser Phe Ala Ala Ser Ser Val Ser Ala Ala Ser Pro Ala Ser Asp Thr
85 90 95

Asp Arg Leu Leu Tyr Ser Thr Ser Met Pro Ala Phe Leu Thr Ala Lys
100 105 110

Arg Asn Lys Asn Pro Gly Asn Leu Asp Trp Ser Asp Asp Gly Cys Ser
115 120 125

Asn Ser Pro Asp Arg Pro Ala Gly Phe Asn Phe Leu Asp Ser Cys Lys
130 135 140

Arg His Asp Phe Gly Tyr Arg Asn Tyr Lys Lys Gln Arg Arg Phe Thr
145 150 155 160

Glu Pro Asn Arg Lys Arg Ile Asp Asp Asn Phe Lys Lys Asp Leu Tyr
165 170 175

Asn Glu Cys Ala Lys Tyr Ser Gly Leu Gln Ser Trp Lys Gly Val Ala
180 185 190

Cys Arg Lys Ile Ala Asn Thr Tyr Tyr Asp Ala Val Arg Ser Phe Gly
195 200 205

Trp Leu
210

<210> 11
<211> 961
<212> DNA
<213> Verticillium tenerum

<220>
<221> CDS
<222> (5)..(628)

<400> 11
caac atg aag acc acc gct gtt ctc tcc ctc gcc atg ctc cag gcc acc 49
Met Lys Thr Thr Ala Val Leu Ser Leu Ala Met Leu Gln Ala Thr
1 5 10 15

tgg gcc tcg ccc gtg gcc aag cgc cag aac gac gtc tcc ctc gtc gac 97
Trp Ala Ser Pro Val Ala Lys Arg Gln Asn Asp Val Ser Leu Val Asp
20 25 30

aac tac atg ttc ggc atc tcg ctg ccc acc ttc tcc aac cac cac tcc 145
Asn Tyr Met Phe Gly Ile Ser Leu Pro Thr Phe Ser Asn His His Ser
35 40 45

aac agg aac ccc cct cgc ctg gac tgg acc acc gac ggc tgc acc tcg 193
Asn Arg Asn Pro Pro Arg Leu Asp Trp Thr Thr Asp Gly Cys Thr Ser
50 55 60

tcg ccc aac aac ccg ctc ggc ttc ccc ttc ctg ccc gcc tgc cac cgc 241
Ser Pro Asn Asn Pro Leu Gly Phe Pro Phe Leu Pro Ala Cys His Arg
65 70 75

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cac gac ttt ggc tac cag aac ttc cgc atc cag agc cgc ttc acc cag      289
His Asp Phe Gly Tyr Gln Asn Phe Arg Ile Gln Ser Arg Phe Thr Gln
80                  85                  90                  95

agc aac aag ctc cgc atc gac gac aag ttc aag gag gac ctc tac cac      337
Ser Asn Lys Leu Arg Ile Asp Asp Lys Phe Lys Glu Asp Leu Tyr His
                100                 105                 110

cag tgc gac ggc cac tgg gcc tgg gtt gcc tgc gct gcc ctc gcc gag      385
Gln Cys Asp Gly His Trp Ala Trp Val Ala Cys Ala Ala Leu Ala Glu
            115                 120                 125

gtc tac tac gcc gcc gtc cgc gcc ttc ggc ggt ggt gac gcc acc ccg      433
Val Tyr Tyr Ala Ala Val Arg Ala Phe Gly Gly Gly Asp Ala Thr Pro
        130                 135                 140

gga cgc atg cac gtc gcc gtc ttc ggc cag acc cag gcc gag cac gac      481
Gly Arg Met His Val Ala Val Phe Gly Gln Thr Gln Ala Glu His Asp
    145                 150                 155

gcc ctc gtc tcc atc tac gag gag aag ctc gcg gcc tac gag gct gcc      529
Ala Leu Val Ser Ile Tyr Glu Glu Lys Leu Ala Ala Tyr Glu Ala Ala
160                 165                 170                 175

gtc gcc gag gcc gag gcc cgc ggc gag atc ccc cac gtc gag gag acc      577
Val Ala Glu Ala Glu Ala Arg Gly Glu Ile Pro His Val Glu Glu Thr
                180                 185                 190

ctc ccc gag gag cct gcc gcc gag gag ccc gcc gcc gag gag gag cag      625
Leu Pro Glu Glu Pro Ala Ala Glu Glu Pro Ala Ala Glu Glu Glu Gln
            195                 200                 205

aag taaacacgag ccccttttag gaccgactag ctcggtgtcg ctgggctagg          678
Lys

ctgagctgag tgacggggag gcacgaaaga gagcaatgca tcagacaggc tggaacatgc   738

ctttgtctga gtgatggatg gacttgatgg acttgatgga cttggatgca tttatgatac   798

cgccagtgtt gactggcaga gcgagcgact tgattttgga tttcttgaaa ggacggatgt   858

cccgaggtgg ataagggatg ccttatcacc aacttcttca tgtatatatt gtactgcgca   918

gagaagcgcg ccccgaaaaa tggattgatt cttgatgaga cgt                     961
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<210> 12
<211> 208
<212> PRT
<213> Verticillium tenerum

<400> 12

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Met Lys Thr Thr Ala Val Leu Ser Leu Ala Met Leu Gln Ala Thr Trp
1               5                   10                  15

Ala Ser Pro Val Ala Lys Arg Gln Asn Asp Val Ser Leu Val Asp Asn
            20                  25                  30

Tyr Met Phe Gly Ile Ser Leu Pro Thr Phe Ser Asn His His Ser Asn
        35                  40                  45

Arg Asn Pro Pro Arg Leu Asp Trp Thr Thr Asp Gly Cys Thr Ser Ser
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50 55 60

Pro Asn Asn Pro Leu Gly Phe Pro Phe Leu Pro Ala Cys His Arg His
65 70 75 80

Asp Phe Gly Tyr Gln Asn Phe Arg Ile Gln Ser Arg Phe Thr Gln Ser
85 90 95

Asn Lys Leu Arg Ile Asp Asp Lys Phe Lys Glu Asp Leu Tyr His Gln
100 105 110

Cys Asp Gly His Trp Ala Trp Val Ala Cys Ala Ala Leu Ala Glu Val
115 120 125

Tyr Tyr Ala Ala Val Arg Ala Phe Gly Gly Gly Asp Ala Thr Pro Gly
130 135 140

Arg Met His Val Ala Val Phe Gly Gln Thr Gln Ala Glu His Asp Ala
145 150 155 160

Leu Val Ser Ile Tyr Glu Glu Lys Leu Ala Ala Tyr Glu Ala Ala Val
165 170 175

Ala Glu Ala Glu Ala Arg Gly Glu Ile Pro His Val Glu Glu Thr Leu
180 185 190

Pro Glu Glu Pro Ala Ala Glu Glu Pro Ala Ala Glu Glu Glu Gln Lys
195 200 205

<210> 13
<211> 29
<212> DNA
<213> Artificial

<220>
<223> TbPLA1 primer

<220>
<221> misc_feature
<222> (4)..(9)
<223> BamHI site

<400> 13
caaggatcca aaatggtcaa gattgctgc 29

<210> 14
<211> 34
<212> DNA
<213> Artificial

<220>
<223> TbPLA2 primer

<220>
<221> misc_feature

<222> (4)..(9)
<223> XhoI site

<400> 14
tgcctcgagt tttttctaga gtggatagat agac 34

<210> 15
<211> 690
<212> DNA
<213> Fusarium venenatum

<220>
<221> CDS
<222> (49)..(597)

<400> 15
cagttttggt tctttccttc cttatccatc acttctagta tcttcaag atg aag ttc 57
Met Lys Phe
1

agc gct acc att ctt tca ctc ctc ccg gca gtt ctc gcc ctg ccc aca 105
Ser Ala Thr Ile Leu Ser Leu Leu Pro Ala Val Leu Ala Leu Pro Thr
5 10 15

ggc gaa gat gca tct gtc tca aag cgc cag agc gtg aac aca gtg aca 153
Gly Glu Asp Ala Ser Val Ser Lys Arg Gln Ser Val Asn Thr Val Thr
20 25 30 35

gat cag ctc ctc ttc agc gtc aca ctc cca caa ttc act gct cgt cgt 201
Asp Gln Leu Leu Phe Ser Val Thr Leu Pro Gln Phe Thr Ala Arg Arg
40 45 50

aac gcc cgt gat cct ccc act gtc gac tgg acc tct gac ggt tgc act 249
Asn Ala Arg Asp Pro Pro Thr Val Asp Trp Thr Ser Asp Gly Cys Thr
55 60 65

tcc tcg ccc gac aac cct ttc ggc ttc cct ttt atc cct gcc tgc aac 297
Ser Ser Pro Asp Asn Pro Phe Gly Phe Pro Phe Ile Pro Ala Cys Asn
70 75 80

cgt cac gac ttt ggc tac cac aac tac cgc gcc cag agc cgc ttc acc 345
Arg His Asp Phe Gly Tyr His Asn Tyr Arg Ala Gln Ser Arg Phe Thr
85 90 95

gtg agc gcc aag tcc cgc atc gac aac aac ttc aag acc gat ttg tac 393
Val Ser Ala Lys Ser Arg Ile Asp Asn Asn Phe Lys Thr Asp Leu Tyr
100 105 110 115

ttc caa tgc caa tcc tcc agt gtt tct ggt gtc tgc aga gca ctt gcc 441
Phe Gln Cys Gln Ser Ser Ser Val Ser Gly Val Cys Arg Ala Leu Ala
120 125 130

gac gtc tac ttc gcc gcg gtt aga gct ttt ggc ggg gat gat gct act 489
Asp Val Tyr Phe Ala Ala Val Arg Ala Phe Gly Gly Asp Asp Ala Thr
135 140 145

cct ggc aag aga gat gag gcc ctt gta aag gag tac gaa aag aag gta 537
Pro Gly Lys Arg Asp Glu Ala Leu Val Lys Glu Tyr Glu Lys Lys Val
150 155 160

gaa gtc tac aac aag ctt gtt gaa gag gct cag aag aag ggt gat ctc 585
Glu Val Tyr Asn Lys Leu Val Glu Glu Ala Gln Lys Lys Gly Asp Leu
165 170 175

cct cgc ctt gac tagagtggtt caaaaagcat tctttgggtt cattgtacat 637
Pro Arg Leu Asp

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180

aaatccttac gatacatgag ttatgataaa tcttaaatgg cgggtgacga gct 690

<210> 16
<211> 183
<212> PRT
<213> Fusarium venenatum

<400> 16

Met Lys Phe Ser Ala Thr Ile Leu Ser Leu Leu Pro Ala Val Leu Ala
1 5 10 15

Leu Pro Thr Gly Glu Asp Ala Ser Val Ser Lys Arg Gln Ser Val Asn
20 25 30

Thr Val Thr Asp Gln Leu Leu Phe Ser Val Thr Leu Pro Gln Phe Thr
35 40 45

Ala Arg Arg Asn Ala Arg Asp Pro Pro Thr Val Asp Trp Thr Ser Asp
50 55 60

Gly Cys Thr Ser Ser Pro Asp Asn Pro Phe Gly Phe Pro Phe Ile Pro
65 70 75 80

Ala Cys Asn Arg His Asp Phe Gly Tyr His Asn Tyr Arg Ala Gln Ser
85 90 95

Arg Phe Thr Val Ser Ala Lys Ser Arg Ile Asp Asn Asn Phe Lys Thr
100 105 110

Asp Leu Tyr Phe Gln Cys Gln Ser Ser Ser Val Ser Gly Val Cys Arg
115 120 125

Ala Leu Ala Asp Val Tyr Phe Ala Ala Val Arg Ala Phe Gly Gly Asp
130 135 140

Asp Ala Thr Pro Gly Lys Arg Asp Glu Ala Leu Val Lys Glu Tyr Glu
145 150 155 160

Lys Lys Val Glu Val Tyr Asn Lys Leu Val Glu Glu Ala Gln Lys Lys
165 170 175

Gly Asp Leu Pro Arg Leu Asp
180